Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
http://dx.doi.org/10.5424/sjar/2014124-6276

Spanish Journal of Agricultural Research 2014 12(4): 1038-1048
ISSN: 1695-971X
eISSN: 2171-9292

RESEARCH ARTICLE

OPEN ACCESS

Dose-dependent effect of a single GnRHa injection on the spawning of meagre (*Argyrosomus regius*) broodstock reared in captivity

Hipolito Fernandez-Palacios[1]*, Dominique Schuchardt[1], Javier Roo[1], Marisol Izquierdo[1], Carmen Hernandez-Cruz[1] and Neil Duncan[2]

[1] *Universidad de Las Palmas de Gran Canaria (ULPGC). Grupo de Investigación en Acuicultura (GIA). Muelle de Taliarte, s/n. 35214 Telde (Las Palmas), Spain.* [2] *IRTA. Sant Carles de la Rápita. Ctra. Poble Nou, km 5.5. 43540 Sant Carles de la Rapita (Tarragona), Spain*

Abstract

The present study aimed to determine the spawning efficacy, egg quality and quantity of captive breed meagre induced with a single gonadotrophin-releasing hormone agonist (GnRHa) injection of 0, 1, 5, 10, 15, 20, 25, 30, 40 or 50 µg kg^{-1} to determine a recommended optimum dose to induce spawning. The doses 10, 15 and 20 µg kg^{-1} gave eggs with the highest quality (measured as: percentage of viability, floating, fertilisation and hatch) and quantity (measured as: total number of eggs, number of viable eggs, number of floating eggs, number of hatched larvae and number of larvae that reabsorbed the yolk sac). All egg quantity parameters were described by Gaussian regression analysis with $R^2 = 0.89$ or $R^2 = 0.88$. The Gaussian regression analysis identified that the optimal dose used was 15 µg kg^{-1}. The regression analysis highlighted that this comprehensive study examined doses that ranged from low doses insufficient to stimulate a high spawning response (significantly lower egg quantities, $p < 0.05$) compared to 15 µg kg^{-1} through to high doses that stimulated the spawning of significantly lower egg quantities and eggs with significantly lower quality (egg viability). In addition, the latency period (time from hormone application to spawning) decreased with increasing doses to give a regression ($R^2 = 0.93$), which suggests that higher doses accelerated oocyte development that in turn reduced egg quality and quantity. The identification of an optimal dose for the spawning of meagre, which has high aquaculture potential, represents an important advance for the Mediterranean aquaculture industry.

Additional key words: fish reproduction; hormonal induction; egg quality.

Introduction

The meagre (*Argyrosomus regius*) is a species that has been identified for the diversification of Mediterranean aquaculture (EATIP Vision, 2012), due to its rapid growth rate and good flesh quality (Monfort, 2010; Duncan *et al*., 2013). Unfortunately, in common with many other fish species meagre exhibit reproductive dysfunctions when held in captivity (Duncan *et al*., 2012; Mylonas *et al*., 2013a,b). Meagre reared and held in captivity have been observed to spawn spontaneously (Mylonas *et al*., 2013b); however, just 2 of 7 females appeared to spawn. Therefore, meagre of both wild (Duncan *et al*., 2012) and reared (Mylonas *et al*., 2013a,b) origin held in captivity appear to exhibit what was described as the most common reproductive dysfunction where oocyte development was arrested in late vitellogenesis and females did not complete oocyte maturation and ovulation (Zohar, 1988, 1989a,b; Peter *et al*., 1993; Zohar & Mylonas, 2001). This dysfunction is often associated with reduced milt volumes and decreased sperm quality in males (Billard, 1986, 1989; Zohar & Mylonas, 2001).

Hormonal treatment has been described as a therapy to overcome these types of reproductive dysfunctions and ensure spawning in captivity (Zohar & Mylonas, 2001). Currently, a widely used technique to induce spawning of broodstock in captivity, has been the use of

* Corresponding author: hipolito.fernandez@ulpgc.es
Received: 20-05-14. Accepted: 27-10-14.

Abbreviations used: EVAc (ethylene vinyl acetate); GnRHa (gonadotrophin-releasing hormone agonists); LH (luteinizing hormone); mGnRHa (mammalian gonadotrophin-releasing hormone agonists); PIT (passive integrated transponder).

gonadotropin releasing-hormone agonists (GnRHa) that stimulate the pituitary and the secretion of luteinizing hormone (LH) for the consequent oocyte maturation, spermiation and spawning. The advantages of the use of GnRHa for the induction of the spawning were suggested to be the stimulation of endogenous gonadotropins and the pituitary-gonad axis and no immunological effects were caused by the peptides (Zohar & Mylonas, 2001). With the development of synthetic agonists (GnRHa), that have a higher potency and longer duration than the native GnRH, use became widespread, both in marine and freshwater species (Mylonas & Zohar, 2001; Zohar & Mylonas, 2001; Mañanos *et al*., 2008).

Wild meagre have been spawned using GnRHa administered as a single injection of 20 μg kg^{-1} and in ethylene vinyl acetate (EVAc) implants of 50 μg kg^{-1} (Duncan *et al*., 2008, 2012). Meagre reared in captivity were also spawned using GnRHa administered in EVAc implants of 46-92 μg kg^{-1} (Mylonas *et al*., 2011, 2013a). In the wild meagre, the single injection gave lower hatching rates and egg size than the implant (Duncan *et al*., 2012) and it was suggested that the single injection protocol could be improved. The spawning patterns between GnRHa treatments were also different, the implants gave more spawns (Duncan *et al*., 2012; Mylonas *et al*., 2011, 2013a), but after initial large spawns, smaller spawns were obtained that counted for approximately 25-30% of all the eggs spawned (Mylonas *et al*., 2011, 2013a). A single GnRHa injection gave a few large spawns and no smaller spawns and Duncan *et al*. (2012) suggested that the differences in spawning pattern between injection and implant were related to the type of ovarian development and spawning strategy. Meagre ovaries have been observed to have all oocyte stages present at one time (Abou Shabana *et al*., 2012; Duncan *et al*., 2012; Schiavone *et al*., 2012; Gil *et al*., 2013) and ovarian development was described as both group-synchronous (Abou Shabana *et al*., 2012; Duncan *et al*., 2012; Schiavone *et al*., 2012) and asynchronous (Gil *et al*., 2013); however, all four studies agreed that meagre ovarian development was characteristic of a batch or group-synchronous spawner. In agreement with these batch spawning characteristics, the wild meagre were induced to spawn for a second time (Duncan *et al*., 2012). One month after the first spawning induction, all females that had been induced to spawn in the first period were observed to again have large vitellogenic oocytes. The eggs spawned from the second induction had similar quality, but the spawning was less frequent and with lower fecundity than after the first spawning induction (Duncan *et al*., 2012). These observations and studies indicate that meagre is a batch spawning species capable of repeated spawns in response to GnRHa induction.

Fornies *et al*. (2001) and Mylonas *et al*. (2003), proposed that a single injection may be more appropriate for the batch spawning species European seabass (*Dicentrarchus labrax*). Duncan *et al*. (2012), suggested that a single injection may also be a more appropriate treatment for meagre and that the few synchronised large spawns obtained with a single injection may be favoured by the industry to stock tanks in a hatchery. This approach may enable hatcheries to use multiple single injections to obtain large spawns from the same females when required for production.

The present study aimed to improve the GnRHa single injection protocol by studying the dose-dependent effect of a single GnRHa injection on the spawning, egg quality and egg quantity to identify the optimal GnRHa dose for inducing spawning from meagre broodstock reared in captivity.

Material and methods

Experimental animals and housing

The meagre used for this study were from an ICCM (Instituto Canario de Ciencias Marinas) stock that was spawned, hatched and reared in captivity. All fish used in the study were marked with passive integrated transponder (PIT) tags (Trovan Ltd., UK), which were read using a tag reader Power Tracker V (Avid, UK). Two similar GnRHa spawning induction experiments (experiments 1 and 2) were made in consecutive years (2009 and 2010) during the natural spawning period of the meagre (Duncan *et al*., 2012, 2013; Mylonas *et al*., 2013a,b). In both experiments broodstock were held in natural environmental conditions in tanks that were supplied with a flow through (400% daily water exchange) of full strength sea water (35 ppt). The temperature during the study period increased from 19.6 to 21.6°C in experiment 1 and was 21 ± 1.3°C in experiment 2.

Determination of maturity status

The fish were anaesthetized using clove oil by firstly making a partial sedation in the holding tank using 10 mL

clove oil per 1000 L and then a complete sedation in an anaesthesia tank with 0.05 mL L^{-1}. Once fully sedated the following measurements were taken: total length and weight. Ovarian biopsies were taken using a catheter with a 1.3 mm internal diameter (Kruuse, Denmark), which was introduced into the genital pore. Each ovarian biopsy, was observed using a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan), and the diameter of one hundred large vitellogenic oocytes were measured. Only females with vitellogenic oocytes greater than 500 µm were selected for the experiments. Milt samples from each male were collected via abdominal pressure. Sperm density was determined in triplicate samples of milt, using a Neubauer hemacytometer (HHH, Germany), and a compound microscope (Leica DM 2500 Wetzlar, Germany) at ×400 magnification. The percentage motility and activity time of the sperm was determined in triplicate samples (Mylonas *et al*., 2004). Maturity status (oocyte diameter or sperm parameters) was only determined once when fish were selected for the experiments. Maturity was not determined immediately before GnRHa treatment, which was completed on different fish at different dates after the start of each experiment (see below for full details on GnRHa treatment). Therefore, maturity status was determined from 4 to 33 days before the fish were treated with GnRHa.

Experiment 1

In experiment 1, the selected females and males had respective mean weights of 8.33 ± 0.97 and 8.47 ± 1.09 kg, and respective mean lengths of 89.16 ± 4.85 and 90.17 ± 4.90 cm. The breeders were randomly distributed in order to stock seven fish (3♀ : 4♂) into each of 6 tanks of 10 m^3 giving five experimental groups (named: 10-(1), 20-(1), 30, 40 and 50) and a control-1 group. The experimental groups were treated with a dose of GnRHa (des-Gly^{10}, [D-Ala^6]-gonadotropin releasing hormone ethylamide; Sigma-Aldrich Co. St. Louis, MO, USA), which was dissolved in saline solution (9 g NaCl/100 mL distilled water) and injected into the dorsal muscle. Injections of GnRHa were made between 08:00 and 09:00 am. The GnRHa treatment consisted of a single injection and the following doses were used for the following experimental groups; 10 µg kg^{-1} for group 10-(1); 20 µg kg^{-1} for group 20-(1); 30 µg kg^{-1} for group 30; 40 µg kg^{-1} for group 40 and 50 µg kg^{-1} for group 50. The bracketed "1" indicates the experimental group was from experiment 1 as some doses were repeated in experiment 2. During the experimental period (14 April-2 June 2009) one female and two males per experimental group were induced each week. Therefore, during the first 3 weeks of the experimental period all females in each experimental group were induced once. Then the fish were induced a second time and a third time following the same weekly procedure and order of fish. However, three females that did not spawn after treatments were replaced to obtain a minimum of one spawn from three different females in each treatment group. Females used as replacements had been held with males in similar conditions to the experimental groups.

Experiment 2

In experiment 2, an almost identical procedure was followed. The same broodstock from experiment 1 was used and the females and males after one year of growth had respective mean weights of 8.93 ± 1.36 and 8.80 ± 1.58 kg, and respective mean lengths of 93.81 ± 5.43 and 93.41 ± 6.64 cm. The breeders were randomly distributed to stock six fish (3♀ : 3♂) into each of eight tanks of 10 m^3 to give six experimental groups (named: 1, 5, 10-(2), 15, 20-(2) and 25) and two control groups. Control-2.1 group was not injected or manipulated and control-2.2 was injected with saline solution and received the same manipulation as the experimental groups. The GnRHa treatment consisted of a single injection and the following doses were used for the following experimental groups: 1 µg kg^{-1} for group 1; 5 µg kg^{-1} for group 5; 10 µg kg^{-1} for group 10-(2); 15 µg kg^{-1} for group 15; 20 µg kg^{-1} for group 20-(2) and 25 µg kg^{-1} for group 25. The bracketed "2" indicates the experimental group was from experiment 2 as some doses were repeated from experiment 1. During the 9-week experimental period one female and one male per experimental group (tank) were induced each week. Therefore, during the first 3 weeks of the experimental period all six fish in each experimental group were induced once. Then the fish were induced a second time (2nd three-week period) and a third time (3rd three-week period) following the same weekly procedure and order of fish. Therefore, over the 9 week period each female was induced three times with an interval between inductions of three weeks. No substitutions of fish were made as all females in the experimental groups spawned.

Determination of egg quantity and quality parameters

To evaluate the effectiveness of the GnRHa inductions the following spawning parameters were determined: percentage of induced females that spawned (No. spawn / No. induction × 100), latency period in experiment 2 (time from the GnRHa injection until the time of the first spawn, egg collectors checked every 15-20 min); mean number of spawns per administration of GnRH; relative fecundity per administration of GnRH (the total number of eggs per injection and per kg of female); percentage viable eggs (subjective assessment where floating eggs were considered viable when morphologically was normal: transparent, perfectly spherical, and with a clear and symmetrical blastomere); percentage floating eggs, percentage fertilised eggs, percentage hatching eggs and percentage of larvae that reabsorbed the yolk sac. The spawning parameters were determined following the methodology described by Fernández-Palacios *et al*. (1995).

Statistical analysis

The results were expressed as mean ± standard deviation of the mean. The data were compared statistically using the analysis of variance (ANOVA) (Sokal & Rohlf, 1996). Once significant differences were detected with ANOVA, differences between means were compared using Duncan's multiple comparison test. The data were analyzed using the program Statgraphics (vers. 5.1 Plus for Windows; Graphic Software Systems Inc. USA). Linear regressions were made with Excel (Microsoft, USA) and the Gaussian regression was explored and made with SigmaPlot vers. 9 (Systat Software Inc., Richmond, CA, USA).

Results

Maturity status

No significant differences were observed in the stage of maturity amongst males or amongst females at the start of each experiment (data not presented). In experiment 1, oocyte diameter ranged from 0.501 ± 0.135 mm in the dose 10-(1) group to 0.535 ± 0.191 mm in the dose 20-(1) group and sperm % motility ranged from 77.32 ± 9.26 in the dose 20-(1) group to 88.95 ± 4.25 in the control-1 group, while sperm density and activity time had an average across groups of 22.4 ± 2.0 × 10^9 and 6.6 ± 0.3 min, respectively. In experiment 2, oocyte diameter ranged from 0.511 ± 0.010 mm in the dose 10-(2) group to 0.537 ± 0.024 mm in the dose 5 group and sperm % motility ranged from 67.64 ± 2.29 in control 2.2 to 80.02 ± 6.84 in the dose 10-(2) group, while sperm density and activity time had an average across groups of 23.4 ± 3.3 × 10^9 and 6.8 ± 0.3 min, respectively.

Spawning response

No spawning was obtained in the control groups. In experiment 1, three females were spawned with each dose; however, only after three females that did not spawn were rejected (Table 1). These rejected females had oocyte sizes greater than 0.5 mm when the experiment was initiated, the female rejected from group 10-(1) was injected three times and the two females rejected from group 50 were injected once and twice (all separated by three weeks). All females in experiment 2 spawned. All injections of 15 μg kg^{-1} gave two spawns, each of the three females was injected three times to give six spawns per female, which was a significantly higher ($p < 0.05$) number of spawns per injection than with any other dose used in experiment 2 (Table 1). Two linear regression analysis described the number of spawns per GnRHa injection against dose used above ($R^2 = 0.73$) and below ($R^2 = 0.95$) the dose of 15 μg kg^{-1} (Fig. 1). The 15 μg kg^{-1} dose group had a latency period of 30.3 ± 1.2 hours that was not different from other doses in experiment 2. However, the latency period decreased significantly ($p < 0.05$) with increasing GnRHa dose (1 to 25 μg kg^{-1}) and the decrease was described by a linear regression analysis ($R^2 = 0.93$) (Fig. 2).

Egg quality and quantity

There were significant differences ($p < 0.05$) between treatments in egg quality (Table 2) and egg quantity (Fig. 3). In general over the two experiments the doses 10, 15 and 20 μg kg^{-1} gave the highest quality and quantity of eggs. In experiment 1, groups 10-(1) and 20-(1) had the highest egg quality in all parameters (% fertilized, % viable, % hatching and % larvae with reabsorbed yolk sac) and in experiment 2, group 15

Table 1. Efficacy of spawning of meagre (*Argyrosomus regius*) induced with different doses (µg kg^{-1}) of a single injection of GnRHa; number of females that responded with number of injections, number of spawns, latency period in experiment 2 and egg yield per kilogram and injection

Experiment	Doses (µg kg^{-1})	Data from females that responded with at least one spawn						
		Nº females that did not respond	Nº injected females that spawned	Nº injections	Nº spawns	Spawns per GnRHa injection	Latency period (h): injection to 1st spawn	Egg yield (egg kg^{-1} inj^{-1})
Exp. 1	10-(1)	1	3	5	9	1.8±0.4	NA	167,203
	20-(1)	0	3	6	9	1.5±0.5	NA	59,505
	30	0	3	5	7	1.4±0.5	NA	102,802
	40	0	3	7	8	1.1±0.4	NA	43,662
	50	2	3	7	5	0.7±0.5	NA	40,665
Exp. 2	1	0	3	9	5	0.5±0.2^{d}	32.15±1.14^{b}	26,129
	5	0	3	9	10	1.1±0.2^{c}	32.33±1.21^{b}	98,188
	10-(2)	0	3	9	14	1.6±0.2^{b}	31.13±1.04^{ab}	160,375
	15	0	3	9	18	2.0±0.0^{a}	30.26±1.24^{a}	247,141
	20-(2)	0	3	9	12	1.3±0.3^{bc}	29.59±1.70^{a}	99,203
	25	0	3	9	11	1.2±0.2^{bc}	29.53±1.68^{a}	130,727

Different superscripts in the same column and experiment indicate significant differences (p<0.05). NA: data not available.

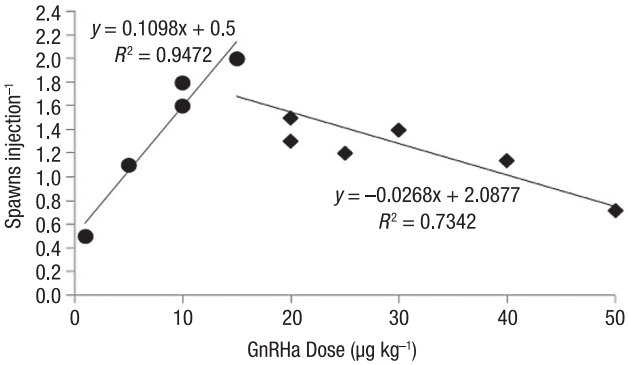

Figure 1. Mean number of spawns per dose (µg kg^{-1}) of a single injection of GnRHa applied to meagre (*Argyrosomus regius*). Regression lines and associated equation and regression coefficients were for doses <15 µg kg^{-1} (circular symbol) and ≥15 µg kg^{-1} (diamond symbol).

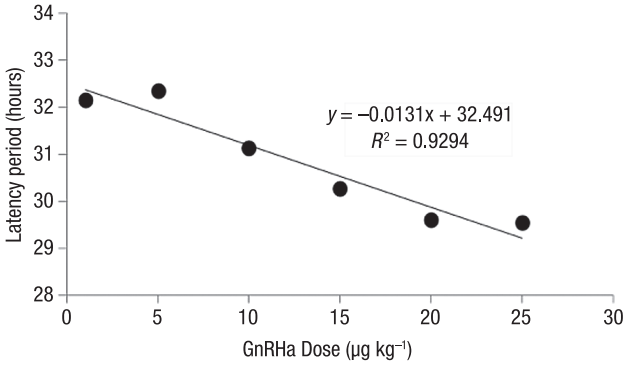

Figure 2. Mean latency period (hours) per dose (µg kg^{-1}) of a single injection of GnRHa applied to meagre (*Argyrosomus regius*) with regression line, associated equation and regression coefficient.

had the highest egg quality over all parameters (Table 2). However, only percentage of viable eggs presented significant differences, in experiment 1, groups 10-(1) (80.73 ± 15.26%) and 20-(1) (87.14 ± 10.66%) had a significantly higher (p < 0.05) percentage of viable eggs and in experiment 2, group 15 (92.41 ± 4.16%) was significantly higher than groups 1, 5, 10-(2) and 25 (Table 2). In experiment 1, group 10-(1) had a higher yield of eggs over all parameters (total number of eggs, number fertilised eggs, number viable eggs, number of hatched larvae and number of larvae with reabsorbed yolk sac), but was only significantly higher than group 40, was significantly higher for selected parameters compared to groups 20 and 50 and was not significantly different compared to group 30 (Fig. 3). In experiment 2, group 15 had a significantly higher yield of eggs in all parameters (Fig. 3). The dose of 15 µg kg^{-1} gave: mean total number of eggs of 123,571 ± 58,848 egg/kg/spawn, mean number of fertilised eggs of 123,053 ± 59,130 eggs/kg/spawn, mean number of viable eggs of 113,862 ± 54,163 eggs/kg/spawn, mean number of hatched larvae of 108,940 ± 53,630 larvae/kg/spawn, mean number of larvae with reabsorbed yolk sac of 100,461 ± 50,685

Table 2. Egg quality parameters: percentage of fertilised eggs, viable eggs, and hatch and percentage of larvae with reabsorbed yolk sac for the different doses (μg kg^{-1}) of a single injection of GnRHa used in experiments 1 and 2 to induce spawning of meagre (*Argyrosomus regius*)

Experiment	Doses (μg kg^{-1})	% Fertilized eggs	% Viable eggs	% Hatching	% Larvae with reabsorbed yolk sac
Exp. 1	10-(1)	95.52±4.81	80.73±15.26^{a}	83.92±18.54	82.44±24.68
	20-(1)	96.97±3.84	87.14±10.66^{a}	89.42±12.95	84.83±10.56
	30	85.91±19.22	40.19±46.54^{b}	64.25±39.26	55.58±42.02
	40	93.83±8.73	25.94±26.19^{b}	66.13±41.49	51.35±37.81
	50	85.22±23.17	43.55±39.71^{b}	60.59±46.15	44.14±44.65
Exp. 2	1	93.70±10.24	50.72±16.27^{d}	88.02±4.58	84.37±9.64
	5	92.90±16.62	71.67±25.71^{bc}	90.71±16.72	89.24±12.31
	10-(2)	98.14±3.64	73.31±18.52^{bc}	91.07±6.12	89.17±17.27
	15	99.33±1.00	92.41±4.16^{a}	95.14±3.63	92.33±6.35
	20-(2)	98.98±2.18	84.46±16.27^{ab}	85.39±16.73	91.51±10.17
	25	98.57±1.71	70.67±14.83^{c}	94.58±5.20	80.58±15.42

Different superscripts in the same column and experiment indicate significant differences ($p < 0.05$).

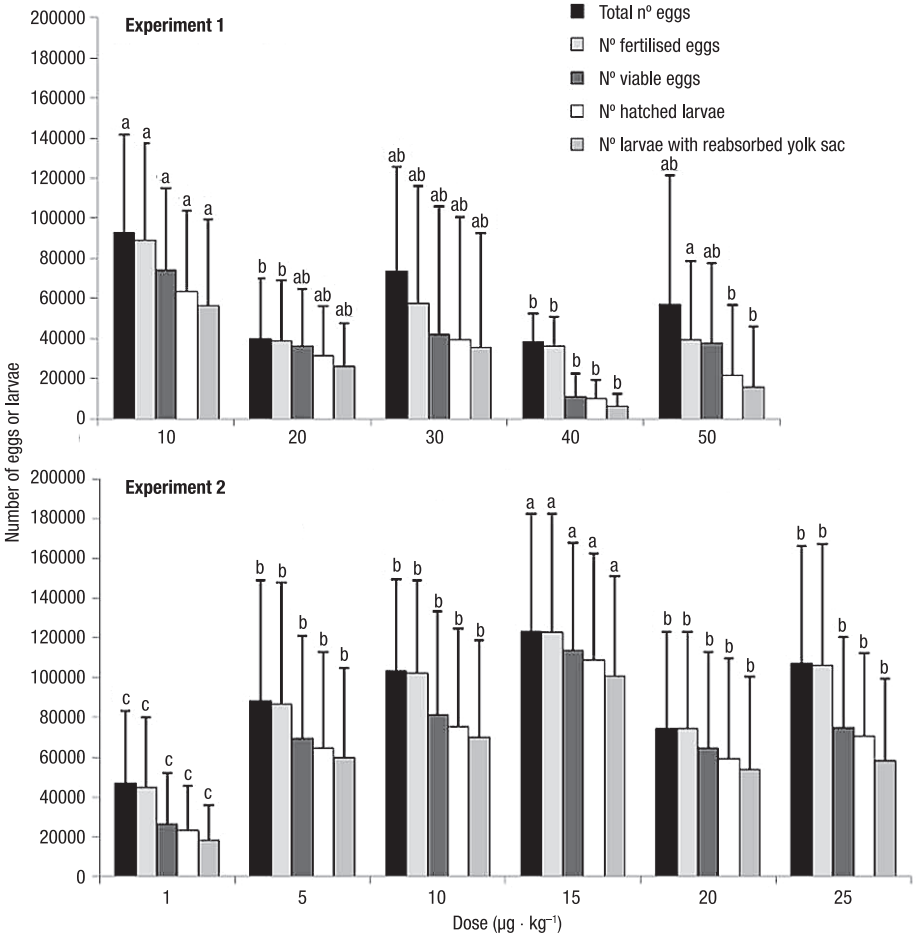

Figure 3. Mean number of eggs and larvae, total number of eggs, number of fertilised eggs, number of viable eggs, number of hatched larvae and number of larvae with reabsorbed yolk sac obtained per kilo of female per spawn after different doses (μg kg^{-1}) of a single injection of GnRHa were applied to meagre (*Argyrosomus regius*) in experiments 1 and 2. Different letters on the same parameter indicate significant differences.

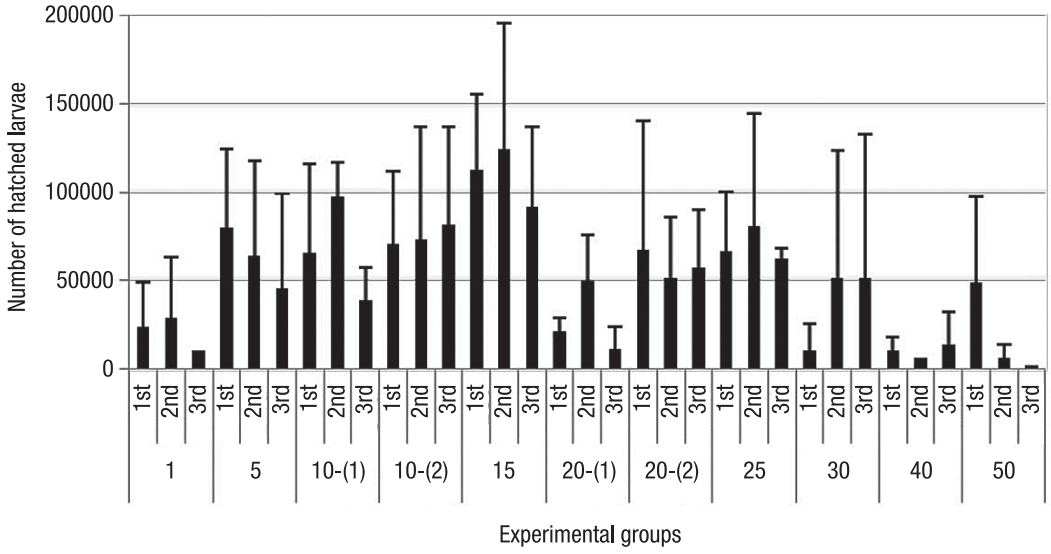

Figure 4. Mean number of larvae obtained per kilo of female per spawn after the first, second and third injection of different doses (μg kg^{-1}) of a single injection of GnRHa were applied to meagre (*Argyrosomus regius*) in experiments 1 and 2. No differences were found amongst the injections within each dose.

Table 3. Gaussian regression equation, R^2 coefficients and constants for the dependent variables, total number of eggs kg^{-1}, number of fertilised eggs kg^{-1}, number of viable eggs kg^{-1}, number of hatched larvae kg^{-1} and number of larvae with reabsorbed yolk sac kg^{-1} against the independent variable GnRHa dose (μg kg^{-1}) that was used to induce spawning of meagre (*Argyrosomus regius*)

Gaussian regression equation[§]

$$y = y_0 + ae^{\left[-0.5\left(\frac{x-x_0}{b}\right)^2\right]}$$

Dependent variable	R^2	x_0	y_0	a	b
Total No. eggs kg^{-1}	0.89	13.2±1.5	57180±12558	79653±59883	2.8±2.7
Nº fertilised eggs kg^{-1}	0.89	13.0±2.0	44732±15602	68344±26844	5.0±2.7
Nº viable eggs kg^{-1}	0.89	13.7±1.6	27844±13323	69523±120604	5.6±2.3
Nº hatched larvae kg^{-1}	0.88	14.5±1.8	15752±16054	67514±19537	7.6±3.0
Nº larvae reabsorbed yolk sac kg^{-1}	0.88	13.8±1.6	15448±13126	64241±17501	6.6±2.5

[§] y = number of eggs or larvae kg^{-1}; x = GnRHa dose, μg kg^{-1}; x_0 = GnRHa dose for maximum egg production.

larvae/kg/spawn. Whilst groups 10-(2) and 20-(2) were only significantly higher in yield compared to group 1.

The different females were induced at intervals of three weeks and no differences were observed in the mean number of hatched larvae obtained amongst the first, second and third injection per female (Fig. 4). No consistent trends were observed of decreasing or increasing number of larvae with each injection, but variation appeared to be greater in groups 1, 5, 10-(1), 20-(1), 30, 40 and 50 compared to groups 10-(2), 15, 20-(2) and 25. Group 15 exhibited the highest consistent mean number of larvae over the first, second and third injection. No differences were observed in the mean number of larvae obtained from the six spawns obtained from each of the first, second and third injections per female (n = 6).

The distribution of means of each egg and larval quantity parameter from the two experiments against doses was described by Gaussian regression analysis and R^2 = 0.89 or R^2 = 0.88 for all parameters (Table 3) and the normal Gaussian distributions was similar to the distribution that described number of hatched larvae against doses (Fig. 5). The Gaussian regression analysis identified that the optimal dose was close to

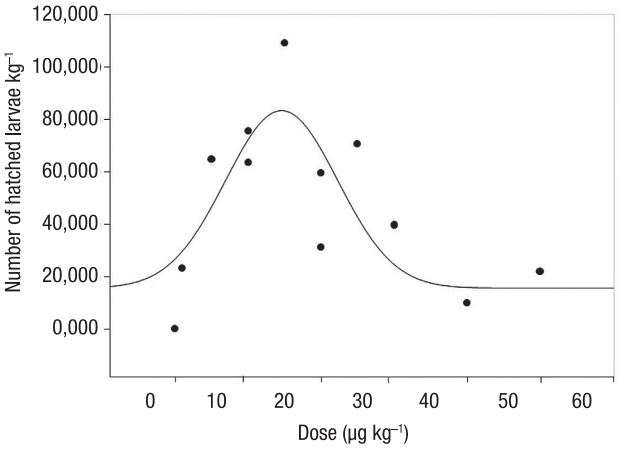

Figure 5. Gaussian regression of mean number of hatched larvae obtained per kilo of female per spawn after different doses (µg kg^{-1}) of a single injection of GnRHa were applied to meagre (*Argyrosomus regius*) in experiments 1 and 2.

15 µg kg^{-1} that was used in the experiments and the Gaussian distributions for the different parameters peaked in a range from 13.0 ± 2.0 for number of fertilised eggs kg^{-1} to 14.5 ± 1.8 for number of hatched larvae kg^{-1} (Table 3) predicting maximum egg or larval production with these doses.

Discussion

The present study has identified that 15 µg kg^{-1} of GnRHa was the optimal single injection dose to induce the spawning of meagre held in the described conditions. The Gaussian regression analysis of egg and larval quantity or yield indicated that 15 µg kg^{-1} was the optimal dose applied; where significant differences were observed the 15 µg kg^{-1} dose exhibited a significantly higher number of eggs and larvae and a significantly higher percentage of egg viability and generally the highest egg quantity (number of eggs and larvae), the highest egg quality (fertilization, viability and hatching); and highest larval quality (larvae with reabsorbed yolk sac) were obtained from the broodstock treated with the 15 µg kg^{-1} dose.

It has been indicated that the aspects to be considered in the development of a GnRHa induced spawning protocol are: (a) stage of ovarian development measured as oocyte diameter (Ibarra & Duncan, 2007; Mañanos *et al*., 2008; Mylonas *et al*., 2010), (b) levels of stress related to husbandry and manipulations to administer the GnRHa (Ibarra & Duncan, 2007; Mañanos *et al*., 2008; Mylonas *et al*., 2010) and (c) the GnRHa dose (Ibarra & Duncan, 2007; Mañanos *et al*., 2008). The GnRHa protocol should identify a minimum oocyte diameter that needs to be attained or surpassed by the maturing fish before successful spawning can be obtained with the administration of GnRHa (Ibarra & Duncan, 2007; Mañanos *et al*., 2008; Mylonas *et al*., 2010). In the present study, oocyte diameter was >0.5 mm in all female meagre at the start of the experiments and 89% of females treated spawned at least once indicating that the minimum required oocyte diameter was surpassed and was not a factor that affected spawning success. The oocyte diameter report in the present study was lower than the 0.55 mm reported for GnRHa induced spawning of wild meagre (Duncan *et al*., 2012) and 0.59 mm reported for meagre reared in captivity (Mylonas *et al*., 2013). However, caution is required in these comparisons as these studies used different procedures to select females. In the present study oocyte diameter was measured at the start of the experiment when the females were selected and the actual oocyte size before hormone application was not known. Duncan *et al*. (2012) and Mylonas *et al*. (2013a) measured oocyte diameter immediately before hormone application. Stress is accepted as one of the principal factors that cause reproductive dysfunctions and that affect a GnRHa spawning protocol (Ibarra & Duncan, 2007; Mañanos *et al*., 2008; Mylonas *et al*., 2010). For example, the stress of capture has been shown to negatively affect reproductive endocrinology, decreasing estrodial and testosterone and oocyte development increasing oocyte atresia in red gurnard (*Chelidonichthys kumu*) (Clearwater & Pankhurst, 1997), female striped trumpeter (*Latris lineate*) (Morehead, 1998) and snapper (*Pagrus auratus*) (Cleary *et al*., 2000). In the present study, the stress from holding conditions and manipulations was the same for all fish in all treatment groups and the sham control (control 2.2) and the high spawning response indicates that the GnRHa protocols were successful in relation to these conditions and the stress that may have been caused by husbandry procedures and manipulation of the fish.

Therefore, the present study determined the effect of a single GnRHa dose on meagre spawning and the wide range of doses used in the two experiments clearly indicate the effect of optimal (15 µg kg^{-1}) and sub-optimal (higher and lower) doses. Optimal GnRHa doses have also been found in similar studies on sea bass (*Lates calcarifer*) (Garcia, 1989), gilthead seabream

(*Sparus aurata*) (Barbaro *et al*., 1997) and spotted rose snapper (*Lutjanus guttatus*) (Ibarra & Duncan, 2007). It has been demonstrated that GnRHa stimulates spawning in fish with arrested maturation by releasing LH from the pituitary, which in turn stimulates oocyte maturation and the fish to spawn (Zohar *et al*., 1995; Mañanos *et al*., 2002; Podhorec *et al*., 2012). Increasing doses (1-20 μg kg^{-1}) of mammalian gonadotrophin-releasing hormone agonists, mGnRHa ([D-Ala^6, Pro^9, NEthylamide]-mGnRH) were positively correlated to levels of circulating LH and ovulation rate in tench (*Tinca tinca*) (Podhorec *et al*., 2011) and high GnRHa doses have been described to accelerate oocyte development (Mylonas *et al*., 1992; Barbaro *et al*., 1997; Mugnier *et al*., 2000; Duncan *et al*., 2003). In the present study latency time from the application of GnRHa to the time eggs were collected decreased linearly (R^2 = 0.93) and demonstrated an accelerating effect of an increasing GnRHa dose. It would appear that the increasing GnRHa dose increases that rate of stimulated development of the arrested oocyte and that the increasing rate of development affected both fecundity and egg quality. In the present study, reducing the dose from the optimal dose (15 μg kg^{-1}) to lower than optimal doses (0, 1, 5 and 10 μg kg^{-1}) exhibited a gradual increase in latency period and decrease in fecundity and egg quality, fertilisation, percentage of viable eggs and percentage hatch. Studies on other species have also found that lower than optimal doses resulted in longer latency period (Yang & Chen, 2004; Wang *et al*., 2009), decreased fecundity (Garcia, 1989; Barbaro *et al*., 1997; Ibarra & Duncan, 2007) and decreased percentage hatch (Ibarra & Duncan, 2007). In the present study, increasing doses to higher than optimal doses (20, 30, 40 and 50 μg kg^{-1}) resulted in a gradual reduction in latency period, fecundity and egg quality, fertilisation, percentage of viable eggs and percentage hatch. Studies on other species have also found that higher than optimal doses resulted in a shorter latency period (Wang *et al*., 2009), decreased fecundity (Garcia, 1989; Ibarra & Duncan, 2007) and decreased egg quality, percentage hatch (Garcia, 1989; Taranger *et al*., 1992; Ibarra & Duncan, 2007), fertilisation (Garcia, 1989; Taranger *et al*., 1992) and percentage of viable eggs (Barbaro *et al*., 1997). The present study appears to confirm that oocyte maturation was accelerated by the administration of GnRHa in a dose-dependent way. At doses lower than the optimal dose, oocyte maturation appeared to be slower and consequentially latency time was longer, fewer oocytes completed maturation reducing fecundity and spawns and a higher proportion of oocytes did not complete maturation correctly and egg quality was reduced. Whilst at doses higher than the optimal dose, oocyte maturation appeared to be faster and consequentially latency time was shorter, accelerated oocyte maturation reduced the number of oocytes correctly completing maturation resulting in lower fecundity, spawns and egg quality.

The present study also observed that repeated doses of 15 μg kg^{-1} administered at intervals of three weeks gave similar mean numbers of hatched larvae after the first, second and third induction. Duncan *et al*. (2012) also observed that a second injection or implant gave a second spawning period in wild meagre, but with a reduced number of spawns and fecundity. The present study did not observe any reduction in fecundity or number of hatched larvae with repeated single injections. The capacity to respond to repeated inductions without any decrease in fecundity and hatching appears to give further confirmation that the meagre has the characteristics of a batch spawner (Abou Shabana *et al*., 2012; Duncan *et al*., 2012; Schiavone *et al*., 2012; Gil *et al*., 2013).

The observation in the present study that meagre have a narrow range of optimal dose of a single injection to induced spawning could be interpreted to be contradictory to studies that found a broad range of GnRHa doses successfully induced spawning in other species and that have concluded that for economic reasons lower doses should be used (Yang & Chen, 2004; Podhorec *et al*., 2012). However, the present study can also be interpreted to have found a broad range of doses (10-30) that have successfully induced spawning. Studies that use a limited range of doses may miss the optimal dose because few or no differences were found especially if all doses tested were close to optimal and an ANOVA group comparison experimental design rather than correlation or regression analysis was used. Shearer (2000) demonstrated that correlation or regression analysis was more appropriate than an ANOVA group comparison experimental design for the analysis of dose-dependent nutritional experiments that aimed to estimate optimal nutrient requirements. The advantages of identifying an optimal dose in the present study were observed in the overall increase in egg production. For example, total egg production increased by 19% in fish induced with 15 μg kg^{-1} compared to fish induced with 10 μg kg^{-1} and 66% in fish induced with 15 μg kg^{-1} compared to fish

induced with 20 μg kg^{-1}. Similarly, number of larvae produced with fully absorbed yolk sac increased by 43% in fish induced with 15 μg kg^{-1} compared to fish induced with 10 μg kg^{-1} and 86% in fish induced with 15 μg kg^{-1} compared to fish induced with 20 μg kg^{-1}. The optimal dose should, therefore, provide a more flexible protocol for application in other conditions compared to a protocol based on a slightly sub-optimal dose.

In conclusion, the present study identified an optimal single injection GnRHa dose for spawning meagre reared in captivity and held in the described conditions. The optimal dose can form the basis of an induction protocol that consisted of a dose of 15 μg kg^{-1} applied to females with an oocyte diameter >0.5 mm held in the described conditions. The study provides the most complete study to date on the effect of varying hormone doses on the spawning of a marine fish species. The wide range of doses tested highlighted the effect of sub-optimal doses (both low and high) and the industrial advantages of using an optimal dose that gave superior egg and larval production compared to sub-optimal doses. The identification of an optimal dose for the spawning of the meagre a species that exhibits excellent aquaculture potential is an important result for the Mediterranean aquaculture industry.

Acknowledgments

This work has been carried out under the project INIA-FEDER-RTA2008-00107-00-00 funded by the *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria* (INIA) coordinated by ND.

References

Abou Shabana NM, Abd El Rahma SH, Al Absawy MA, Assem SS, 2012. Reproductive biology of *Argyrosomus regius* (Asso, 1801) inhabiting the south eastern Mediterranean Sea, Egypt. Egypt J Aquac Res 38: 147-156.

Barbaro A, Francescon A, Bozzato G, Merlin A, Belvedere P, Colombo L, 1997. Induction of spawning in gilthead seabream, *Sparus aurata* L., by a long-acting GnRH agonist and its effects on egg quality and daily timing of spawning. Aquaculture 197: 349-359.

Billard R, 1986. Spermatogenesis and spermatology of some teleost fish. Reprod Nutr Dev 26: 877-920.

Billard R, 1989. Endocrinology and fish culture. Fish Physiol Biochem 7: 49-58.

Clearwater SJ, Pankhurst NW, 1997. The response to capture and confinement stress of plasma cortisol, plasma sex steroids and vitellogenic oocytes in the marine teleost, red gurnard. J Fish Biol 50: 429-441.

Cleary JJ, Pankhurst NW, Battaglene SC, 2000. The effect of capture and handling stress on plasma steroid levels and gonadal condition in wild and farmed snapper *Pagrus auratus* (Sparidae). J World Aquacult Soc 31: 558-569.

Duncan NJ, Rodriguez M, Alok D, Zohar Y, 2003. Effects of controlled delivery and acute injections of LHRHa on bullseye puffer fish (*Sphoeroides annulatus*) spawning. Aquaculture 218: 626-635.

Duncan N, Estevez A, Padros F, Aguilera C, Montero FE, Norambuena F, Carazo I, Carbo R, Mylonas CC, 2008. Acclimation to captivity and GnRHa-induced spawning of meagre (*Argyrosomus regius*). Cybium 32: 332-333.

Duncan N, Estevez A, Porta J, Carazo I, Norambuena F, Aguilera C, Gairin I, Bucci F, Valles R, Mylonas CC, 2012. Reproductive development, GnRHa-induced spawning and egg quality of wild meagre (*Argyrosomus regius*) acclimatised to captivity. Fish Physiol Biochem 38: 1273-1286.

Duncan NJ, Estevez A, Fernández-Palacios H, Gairin I, Hernández-Cruz CM, Roo J, Schuchardt D, Vallés R, 2013. Aquaculture production of meagre (*Argyrosomus regius*): hatchery techniques, ongrowing and market. In: Advances in aquaculture hatchery technology, vol 242 (Allan G & Burnell G, eds). Woodhead Publishing Limited, Cambridge UK, pp: 519-541.

EATIP, 2012. The future of European Aquaculture: a strategic agenda for research and innovation. Available in www.eatip.eu. [18 June 2013].

Fernández-Palacios H, Izquierdo MS, Robaina L, Valencia A. Salhi M, Vergara JM, 1995. Effect of n-3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata* L.). Aquaculture 132: 325-337.

Fornies MA, Mañanos E, Carrillo M, Rocha A, Laureau S, Mylonas CC, Zohar Y, Zanuy S, 2001. Spawning induction of individualized European sea bass females (*Dicentrarchus labrax*) using different GnRHa-delivery systems. Aquaculture 202: 221-234.

García LMB, 1989. Dose-dependent spawning response of mature female sea bass, *Lates calcarifer* (Bloch), to pelleted luteinizing hormone-releasing hormone analogue (LHRHa). Aquaculture 77: 85-96.

Gil MM, Grau A, Basilone G, Ferreri R, Palmer M, 2013. Reproductive strategy and fecundity of meagre *Argyrosomus regius* Asso, 1801 (Pisces: Sciaenidae): implications for restocking programs. Sci Mar 77: 105-118.

Ibarra-Castro L, Duncan NJ, 2007. GnRHa-induced spawning of wild-caught spotted rose snapper *Lutjanus guttatus*. Aquaculture 272: 737-746.

Mañanos E, Carrillo M, Sorber LA, Mylonas CC, Asturiano JF, Bayarri MJ, Zohar Y, Zanuy S, 2002. Luteinizing hormone and sexual steroid plasma levels after treatment of European sea bass with sustained-release delivery systems for gonadotropin-releasing hormone analogue. J Fish Biol 60: 328-339.

Mañanos E, Duncan NJ, Mylonas CC, 2008. Reproduction and control of ovulation, spermiation and spawning in cultured fish. In: Methods in reproductive aquaculture (Cabrita E, Robles V, Herraez P, eds). CRC Press, Boca Raton, FL, USA, pp: 1-83.

Monfort MC, 2010. Present market situation and prospects of meagre (*Argyrosomus regius*), as an emerging species in Mediterranean aquaculture, studies and reviews. General Fisheries Commission for the Mediterranean No. 89, FAO, Roma, pp: 1-28.

Morehead DT, 1998. Effect of capture, confinement and repeated sampling on plasma steroid concentrations and oocyte size in female striped trumpeter *Latris lineata* (Latrididae). Mar Freshwater Res 49: 373-377.

Mugnier C, Guennoc M, Lebegue E, Fostier A, Breton B, 2000. Induction and synchronisation of spawning in cultivated turbot (*Scophthalmus maximus* L.) broodstock by implantation of a sustained-release GnRH-a pellet. Aquaculture 18: 241-255.

Mylonas CC, Zohar Y, 2001. Use of GnRHa-delivery systems for the control of reproduction in fish. Rev Fish Biol Fisher 10: 463-491.

Mylonas CC, Hinshaw JM, Sullivan CV, 1992. GnRHa-induced ovulation of brown trout (*Salmo salar*) and its effects on egg quality. Aquaculture 106: 379-392.

Mylonas CC, Sigelaki I, Divanach P, Mañanos E, Zanuy S, Afonso-Polyviou A, 2003. Multiple spawning of individual European sea bass (*Dicentrarchus labrax*) after repeated injections of GnRHa, and evaluation of egg quality. Aquaculture 221: 605-620.

Mylonas CC, Kyriakou Y, Sigelaki I, Georgiou G, Stephanou D, Divanach P, 2004. Reproductive biology of the shi drum (*Umbrina cirrosa*) in captivity and induction of spawning using GnRHa. Isr J Aquacult-Bamid 56: 75-92.

Mylonas CC, Fostier A, Zanuy S, 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen Comp Endocr 165: 516-534.

Mylonas CC, Mitrizakis N, Sigelaki I, Papadaki M, 2011. Spawning kinetics of individual female meagre (*Argyrosomus regius*) after treatment with GnRHa implants. Proc 9th Int Symp on Reproductive Physiology of Fish, Cochin, India. 9-14 August 2011.

Mylonas CC, Mitrizakis N, Castaldo CA, Cerviño CP, Papadaki M, Sigelaki I, 2013a. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity II. Hormonal induction of spawning and monitoring of spawning kinetics, egg production and egg quality. Aquaculture 414-415: 318-327.

Mylonas CC, Mitrizakis N, Papadaki M, Sigelaki I, 2013b. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual reproductive cycle. Aquaculture 414-415: 309-317.

Peter RE, Lin HR, Van Der Kraak G, Little M, 1993. Releasing hormones, dopamine antagonists and induced spawning. Recent Adv Aquacult 4: 25-30.

Podhorec P, Socha M, Sokolowska-Mikolajczyk ME, Drozd B, Policar T, Stejskal V, Kouril J, 2011. Effective dose of mGnRHa for induction of ovulation in tench (*Tinca tinca* L.). Aquaculture 319: 184-187.

Podhorec P, Socha M, Sokolowska-Mikolajczyk ME, Policar T, Svinger VW, Drozd B, Kouril J, 2012. Dopamine control of LH release in the tench (*Tinca tinca*). Gen Comp Endocrinol 175: 34-38.

Schiavone R, Zilli L, Storelli C, Vilella S, 2012. Changes in hormonal profile, gonads and sperm quality of *Argyrosomus regius* (Pisces, Scianidae) during the first sexual differentiation and maturation. Theriogenology 77: 888-898.

Shearer KD, 2000. Experimental design, statistical analysis and modelling of dietary nutrient requirement studies for fish: a critical review. Aquacult Nutr 6: 91-102.

Sokal RR, Rolf J, 1996. Biometría: Principios y métodos estadísticos en la investigación biológica. Editorial Blume, Madrid, 832 pp.

Taranger GL, Stefansson SO, Hansen T, 1992. Advancement and synchronization of ovulation in atlantic salmon (*Salmo salar* L) following injections of LHRH analog. Aquaculture 102: 169-175.

Wang Y, Hu M, Wang W, Li X, Cheung SG, Shin PKS, Song L, 2009. Effects of GnRHa (D-Ala^6, Pro^9-NEt) combined with domperidone on ovulation induction in wild loach *Misgurnus anguillicaudatus*. Aquaculture 291: 136-139.

Yang Z, Chen Y, 2004. Induced ovulation in obscure puffer *Takifugu obscurus* by injections of LHRHa. Aquac Int 12: 215-223.

Zohar Y, 1988. Gonadotropin releasing hormone in spawning induction in teleosts: basic and applied considerations. In: Reproduction in fish: basic and applied aspects in endocrinology and genetics (Zohar Y & Breton B, eds). INRA Press, París, pp: 47-62.

Zohar Y, 1989a. Fish reproduction: its physiology and artificial manipulation. In: Fish culture in warm water systems: problems and trends (Shilo M & Sarig S, eds). CRC Press, Boca Raton, pp: 65-119.

Zohar Y, 1989b. Endocrinology and fish farming: aspects in reproduction growth, and smoltification. Fish Physiol Biochem 7: 395-405.

Zohar Y, Mylonas CC, 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture 197: 99-136.

Zohar Y, Harel M, Hassin S, Tandler A, 1995. Gilt-head sea bream (*Sparus aurata*). In: Broodstock management and egg and larval quality (Bromage NR & Roberts RJ, eds). Blackwell, Oxford, UK, pp: 94-117.